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בקשה לפטנט
Application For Patent

לשימוש הלשכה
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אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
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בעל אמצאה מכח היותי ממציאה being inventor ששמה הוא
Owner, by virtue of of an invention the title of which is

(בעברית) (Hebrew)
מעכבים לפרוטאזות של פיקורנווירוס

(באנגלית) (English)
Inhibitors for picornavirus proteases

מבקש בזאת כי ינתן לי עליה פטנט hereby apply for a patent to be granted to me in respect thereof.

* דרישת דין קדימה Priority Claim			* בקשת פטנט מוסף — Appl. for Patent of Addition	* בקשת חלוקה — Application of Division
מדינת האיגוד Convention Country	תאריך Date	מספר/סימן Number/Mark	* לבקשה/לפטנט to Patent/Appl.	מבקשת פטנט from application
			No. מס' dated מיום	No. מס' dated מיום

* יפוי כח: עוד יוגש P.O.A.:

הוגש בענין קודם filed on a previous case

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היום 13th בחודש June שנת 1996 This of the year

חתימת המבקש
Signature of Applicant
For the Applicants,
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By: -

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מעכבים לפרוטאזות של פיקורנווירוס

Inhibitors for picornavirus proteases

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FIELD OF THE INVENTION

The present invention concerns novel inhibitors of the 3C and 2A proteases of the *picornavirus* and pharmaceutical compositions comprising said inhibitors.

BACKGROUND OF THE INVENTION

Picornaviruses are single-stranded positive RNA viruses that are encapsulated in a protein capsid. These viruses cause a wide range of diseases in man and animal including common cold, poliomyelitis, hepatitis A, encephalitis, meningitis and foot-and-mouth disease, as well as disease in plants such as the potty disease in potatoes. After inclusion into the host cell the picornaviral RNA is translated into a 247-kDa protein that is co-and post translationally cleaved, yielding 11 mature proteins. The 2A and 3C proteolytic enzymes which are part of the picornaviral self polyproteins are responsible for these cleavages. The 2A protease cleaves co-translationally between the structural and non-structural proteins and the 3C protease cleaves post-translationally the remaining cleavage sites except one.

Having been recognized as important proteins in the picornaviral life cycle, by virtue of being responsible for its maturation, the 3C and 2A proteases have been a prime target for extensive structural and mechanistic investigations during the last few years and their mechanism and structural features have been

determined (Kreisberg *et al*, *Organic Reactivity: Physical and Biological Aspects*, 110-122 (1995)).

Attempts to produce agents against picornaviruses are directed towards finding inhibitors for the 3C and 2A proteases, due to the fact that inhibiting these proteases will avoid production of new virons since there are no native cellular proteases which can replace the cleavage activity of the viral proteases. Therefore, finding an efficient inhibitor against 3C and/or 2A picornavirus proteases will lead to the production of an anti-viral pharmaceutical composition against a large number of viral diseases, both in man and in animal.

Site-directed mutagenesis studies (Cheah K.C. *et al*, *J. Biol. Chem.*, **265** (13), 7187-7189 (1990)) confirmed by X-ray studies (Matthews *et al*, *Cell*, 77: 761-771, (1994)) led to the finding that the catalytic site of 3C is composed of the following amino acids: Cys in position 146, Glu/Asp in position 71 and His in position 70. These three amino acids in the catalytic site of the 3C enzyme constitute a hybrid between the amino acids at the catalytic site of cyteine proteases and serine proteases.

The first agent found as an inhibitor of the 3C protease is *thysanon*, an antibiotic compound obtained from *Thysanphora penicilidies* (Singh *et al*, *Tetrahedron Lett.*, **32**: 5279-82 (1991)). However, this compound was not developed into a pharmaceutical composition, since it was found to be an efficient inhibitor of the elastase enzyme present in erythrocytes.

Two additional antibiotic compounds, of fungal origin, termed *citrinin hydrate* and *radicinin* were obtained by screening microbial extracts (Kadan *et al*, *J. Antibiotics* 7: 836-839 (1994)). These novel two compounds showed a lower level of inhibition than thysanon. The same year a new compound termed *kalafungin*, which is also an antibiotic compound, was discovered by structural comparison to radicinin, and was found to be a better inhibitor, by three orders of magnitude, than radicinin and citrinin hydrate (McCall *et al*, *Biotechology*, **12**: 1012-1016 (1994)).

The cells of the slime mold *Dictyostelium discoideum* grow as a single amoeba, but when starved they aggregate in response to propagating waves of

cyclic-AMP to form a multi-cellular organism which transforms itself into a migrating 'slug'. Within this slug there is a simple spacial pattern of cell differentiation with prestalk cells in the anterior and prespore cells in the posterior. This pattern reflects the final stalk/spore propagation of the mature fruiting body. Stalk-cell differentiation *in vitro* can be induced by Differentiation Inducing Factor 1 (DIF1), a factor released by developing cells (Town *et al*, *Nature*, **202**: 717-719 (1976); Brookman *et al*, *Dev. Biol.*, **91**: 191-196 (1982)). DIF1 has been defined as 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl) hexan-1-one (Morris *et al*, *Nature*, **328**: 811-814 (1987)). Recently, it has been found that DIF1 induces differentiation of murine and human undifferentiated leukaemia so that in concentrations of 2mg/ml (6.5 mM), DIF1 induced the differentiation in murine erythroleukemia B8 cells and human leukaemia K562 cells into haemoglobin-synthesizing erythrocyte-like cells (Ashaki *et al*, *Bich. Biophy. Res. Com.*, **208** (3): 1036-1039 (1995)).

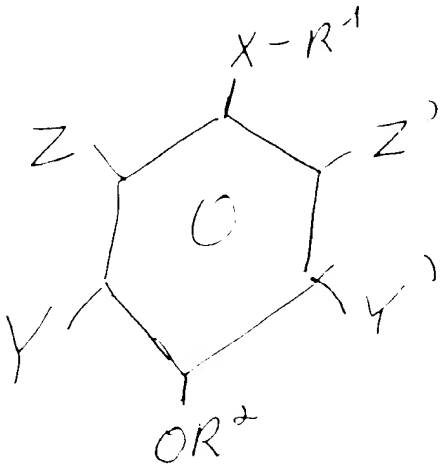
SUMMARY OF THE INVENTION

The present invention is based on the surprising finding that DIF1 is a specific inhibitor of the picornavirus 3C protease, and does not inhibit other proteases normally present in the cell. The present invention is based on the further findings that derivatives of DIF1 are more effective inhibitors than the native compound.

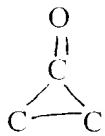
The advantage of using the DIF1 as an inhibitor of picornavirus proteases, as compared to prior art inhibitors of 3C protease resides in the fact that prior art inhibitors constitute either 3 (thysanon, kalafungin) or 2 (radicinin, citrinin hydrate) attached rings, which give the construct a certain rigidity, while DIF1 comprises a single substituted ring, having a flexible hydrophobic tail, which may accommodate into the binding-groove of the 3C protease more easily. Furthermore, the hydrophobic tail may be tailored specifically to match the binding groove of species-specific enzymes of the 3C protease derived from different viruses, rendering the inhibitor specific to one or more types of viruses, and enabling productions of pharmaceutical compositions suitable for the treatment of one

specific disease. It has previously been found (Yiu S.F. *et al*, *Virology*, 1, 615-625 (1991)) that inhibitors of 3C picornavirus protease are also efficient inhibitors of 2A picornavirus protease, so that the inhibitors of the present invention may also be used to inhibit the 2A protease.

Thus, the present invention is directed to specific inhibitors of picornavirus 3C and/or 2A proteases of the formula:



I

Wherein X is C=O, S=O,  C, C=S, C-N-OR, or  ;

R^1 is a hydrocarbon group, an oligopeptide of 3 to 12 amino acids, or a small organic molecule prepared by peptidomemetic, having the same binding properties as said oligopeptide;

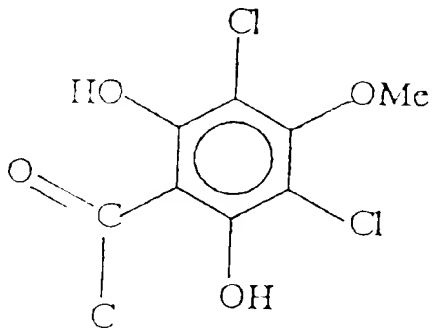
R^2 is H, C_1-C_{12} substituted or unsubstituted hydrocarbon, or an oligopeptide of 3 to 12 amino acids;

Z and Z^1 are each independently OH, SH or one of Z and Z^1 may also be H;

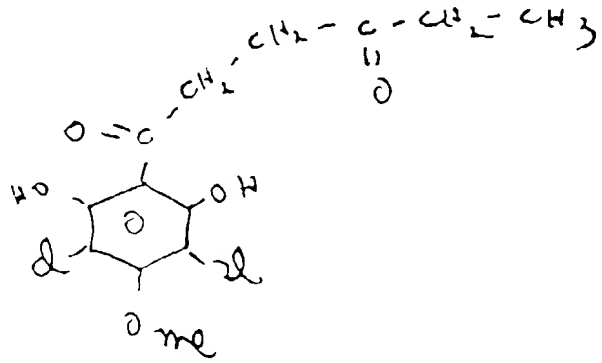
Y and Y^1 are the same or different electron withdrawing groups or one of Y and Y^1 may also be H.

Examples of specific inhibitors of the picornavirus 3C or 2A protease are compounds of the following formulae II - IV:

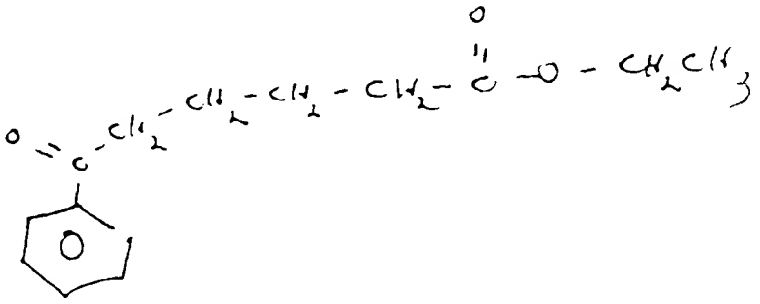
DIF1



II



III



IV

The electrons withdrawing group may suitably be, e.g.: halogen, CN, CF_3, aldehyde, NO_2, SO_3H, COOH and the like, or a phenyl substitute, by one or more of halo, CN, CF_3, NO_2, SO_3H, COOH or aldehyde.

R^2 is a straight or branched C_1-C_{18} hydrocarbon chain, preferably a C_1-C_6 alkyl or R^2 is H. Optionally the hydrocarbon of R^2 bears a label such as a

radioisotope, a fluorescent label, or a member of the pair biotin/avidin and the like. These detectable labels will enable specific determination of the presence of 3C or 2A picornavirus proteases in a sample indicative of a picornaviral infection. R^2 may also bear a cytotoxic moiety capable of destroying the picornavirus inducing cell, or a specific marker which serves as an indicator to various cellular enzymes or molecules, that the molecule to which it is attached should be dègraded. In such a case the specific inhibitor of the invention is used to target the cytotoxic moiety, or degradation marker, specifically to the picornavirus.

Alternatively, R^2 may be an oligopeptide of 3 to 12 amino acid units, preferably an oligopeptide capable of binding to the 3C binding groove at the region from the proline residue (the cleavage site) in the binding groove towards the C-terminal direction of the protease. Various options of R^2 suitable as substitutes in compounds against specific viruses are depicted in Fig. 2. The sequence of R^2 may also be a fragment of the sequences depicted in Fig. 2.

R^1 is the moiety that renders the inhibitor of the invention specific to the picornavirus species, whose 3C protease is to be inhibited, since this is the region that binds to the protein-binding groove of the enzyme, beginning at the amino acid proline present in the binding groove and extending toward the N-terminal direction of the 3C protein.

Thus, R^1 may be tailored specifically to bind to a desired 3C enzyme, giving the inhibitor its virus specificity.

In accordance with the first embodiment of the invention R^1 is a hydrophobic group, for example: a straight, branched, mono- or bicyclic hydrocarbon group having up to 12 carbon atoms, which may be saturated or unsaturated and optionally substituted by oxo, carbanoyl, alkoxycarbonyl, phenyl, alkanoyl or carboxyalkyl groups.

In accordance with a second embodiment of the invention R^1 is an oligopeptide of 3 to 12 amino acid units capable of specifically recognizing and binding the binding-groove of the species-specific 3C protease against which the inhibitor is directed, since the binding cleft of each 3C protease differs slightly form one type of picornavirus to the other.

Generally speaking, the oligopeptide should be able to mimic the amino acid sequence of the viral proteins to which the 3C protease binds; some of these amino acids are conserved and common to all picornaviruses, while others vary between the sub-families, and species.

Examples of R^1 sequences suitable in accordance with the second embodiment of the invention are specified in fig. 1, wherein each sequence is depicted next to the name of the virus which 3C protease the respective R^1-carrying compound may inhibit.

R^1 may also be a fragment of at least 3 amino acids of the sequences specified in Fig. 1.

Other examples, of suitable amino sequences of various picornaviruses may be constructed according to the teachings of Coldingley *et al J. Biol Chem.*, **265** (16): 9062-9065 (1990).

Suitable oligopeptides of 3 to 12 amino acids may be screened, by immobilizing an appropriate 3C picornavirus protease, for example by attaching it to beads and then determining which peptides were able to specifically bind to the immobilized proteases. Such oligopeptides are suitable for serving as R^1 in the inhibitor of the second embodiment of the invention.

By a third embodiment of the invention the R^1 may be a small organic molecule, for example selected from one of the molecules stipulated in Fig. 3. prepared by peptidomemetic methods, which has similar binding properties to the binding groove as the oligopeptide of the second embodiment of the invention. Methods for preparing peptidomemetic compounds are well known in the art and are specified in Quantitative Drug Design C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992).

The inhibitors of the present may be synthesized according to standard procedures for example those specified in Magento *et al*, *Bio. Chem. J.*, **250**, 23-28 (1988).

As will be no doubt appreciated by the person versed in the art, Formula I above covers a large number of possible compounds, some of which are more effective inhibitors of 3C and 2A proteases than others.

In order to determine which of the compounds are suitable as inhibitors the compounds may be screened for inhibitory activities according to one of the following assays:

Assays for screening picornaviral proteases inhibitors

I G. M Birch et al (G.M. Birch et al, *Protein Expression and Purification*, 6, 609-618 (1995)) have developed a continuous fluorescence assay to determine kinetic parameters and for screening potential HRV14 3C inhibitors. the assay consists of the consensus peptide for assaying rhinoviruses attached to fluorescence groups on its N and C terminal, using anthranilic acid donor group on one side of the scissile bond (Gln/Gly) and p-NO2-Phe acceptor group at the P4 position. the substrate peptide consists on the following sequence:

Anthranilic acid Anc)-Thr-Leu-Phe-Gln-Gly-Pro-Val-(p-NO2)-Phe-Lys. This substrate mimics the natural peptidic substrate. A plot of relative fluorescence intensity vs. time shows the linear time dependent increase in fluorescence as the substrate is cleaved. the time dependant plot allowed continuous monitoring of the reaction. For screening, the intensity of fluorescence in each well measures the inhibitory effect of each inhibitor, and allows fast screening of many inhibitors, each occupying a single well.

II B.A Heinz et al (*Antimicrobial Agents and Chemotherapy*, 267-270 (1996)) developed an assay method for measuring HIV protease activity and inhibition. this assay uses the following substrate: biotin-Arg-Ala-Glu-Leu-Gln-Gly-Pro-Tyr-Asp-Glu-Lys-fluorescein isocyanate. Anti-viral activity is tested in HeLa cell monolayers grown in 96-well plates in minimum essential medium containing Earle's balanced salt solution and other components. To quantitate anti-viral activity, 50μM of freshly prepared XTT-PMS medium is added to each well and the plates are incubated at 37° for 2-3 hours. Color development, indicating the presence of metabolically active cells, is detected spectrophotometrically (A450). The concentration of compound required to prevent 50% of the cytopathic effect (50% inhibitory

concentration (IC_{50})) is calculated from the linear portion of each dose-response curve and is indicative of the inhibitory efficiency of the compound. Compound toxicity (50% toxic concentration TC_{50}) is recorded as the concentration of drug that resulted in a 50% cytopathic effect in uninfected cell controls.

III Another assay developed by J. Owen McCall et al (Bio/Technology, **12**, 1012-1016 (1994)) measures on top of inhibitory effects of the candidate inhibitors, also their capability to enter into cells, so that a high capacity screen for compounds that inhibit the 3C protease of HRV-1b was developed. The assay uses a recombinant strain of E-coli expressing both the protease modified to contain the minimal protease cleavage and the tetracycline site resistance. Cultures growing in microliter plates containing tetracycline are treated with potential inhibitors and stimulant. Culture with no inhibition of the 3C protease, show reduced growth due to cleavage of the essential gene product. Normal growth is seen only in cultures that contains an effective 3C protease inhibitor.

IV We have developed an assay, based on a fused protein constructed of the 3C enzyme with its substrate region fused to DHFR. The cleavage of the fused protein by external 3C enzyme (type 1A) is monitored by gel-electrophoresis. The amount of cleavage is proportional to the growing low molecular weight proteins (3C+DHFR) observed on the gel, relative to the high molecular weight of the fused protein. (Arad et al., manuscript in preparation). Inhibition is determined by a decrease in the accumulation of low molecular weight proteins as compared to control.

The present invention further concerns a pharmaceutical composition for the treatment of picornavirus infections comprising a pharmaceutically acceptable carrier and as an active ingredient a therapeutically effective amount of the compound of formulae I to IV.

The pharmaceutical compositions of the invention are suitable for the treatment of: common colds, allergic rhinitis, poliomyelitis, hepatitis-A, encephalitis, meningitis, foot-and-mouth disease and encephamiocarditis.

The inhibitors of the present invention selectively bind to the picornaviral proteases, essentially in a similar manner as the virally coded natural substrate of the proteases, and compete with the substrates for proteases. This competition serves to inhibit viral maturation and thus to inhibit disease progression *in vivo*.

Thus the present invention further provides a method for treatment of a picornaviral infection by administrating to a subject, in need of such treatment, a pharmaceutically acceptable amount of a compound of formulae I to IV, optionally together with a pharmaceutically acceptable carrier.

Dosage units of the active ingredient may be selected by procedures routine to a person skilled in the art.

Pharmaceutically acceptable carriers are well known in the art and are disclosed, for instance, in *Sprowl's American Pharmacy*, Ditter, L. (ed.), J.B. Lippincott Co., Philadelphia, 1974, and *Remington's Pharmaceutical Sciences*, Gennaro, A. (ed.), Mack Publishing Co., Easton, Pennsylvania, 1985.

Pharmaceutical compositions of the compounds of the present invention, or of pharmaceutically acceptable salts thereof, may be formulated as solutions or lyophilized powders for parenteral administration. Powders may be reconstituted by addition of a suitable diluent or other pharmaceutically acceptable carrier prior to use, The liquid formulation is generally a buffered, isotonic, aqueous solution, but a lipophilic carrier, such as propylene glycol optionally with an alcohol, may be more appropriate for compounds of this invention. Examples of suitable diluents are normal isotonic saline solution, standard 5% dextrose in water of buffered sodium or ammonium acetate solution. Such a formulation is especially suitable for parenteral administration, but may also be used for oral administration or contained in a metered dose inhaler of nebulizer for insufflation. It may be desirable to add excipients such as ethanol, polyvinylpyrrolidone, gelatin, hydroxy cellulose, acacia, polyethylene glycol, mannitol, sodium chloride or sodium citrate.

Alternately, the compounds of the invention may be encapsulated, tableted or prepared in an emulsion or syrup for oral administration. Pharmaceutically acceptable solid or liquid carriers may be added to enhance or stabilize the composition, or to facilitate preparation of the composition. Liquid carriers include syrup, soy bean oil, peanut oil, olive oil, glycerin, saline, ethanol, and water. Solubizing agents, such as dimethylsulfoxide, ethanol or formamide, may also be added. Carriers, such as oils, optionally with solubizing excipients, are especially suitable. Oils include any natural or synthetic non-ionic water-immiscible liquid, or low melting solid, which is capable of dissolving lipophilic compounds. Natural oils, such as triglycerides are representative, In fact, another aspect of this invention is a pharmaceutical composition comprising a compound of formula (I) and an oil.

Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, magnesium stearate or stearic acid, talc, pectin, acacia, agar or gelatin. Solubilizing agents, such as dimethylsulfoxide or formamide, may also be added. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax. The pharmaceutical preparations are made following the conventional techniques of pharmacy involving milling, mixing, granulating, and compressing, when necessary, for tablet forms; or milling, mixing and filling for hard gelatin capsule forms. When a liquid carrier is used, the preparation will be in the form of a syrup, elixir, emulsion or an aqueous or non-aqueous suspension. Such a liquid formulation may be administered directly p.o. or filled into a soft gelatin capsule.

For rectal administration, a pulverized powder of the compounds of this invention may be combined with excipients such as cocoa butter, glycerin, gelatin or polyethylene glycols and molded into a suppository. The pulverized posers may also be compounded with an oily preparation, gel, cream or emulsion, buffered or unbuffered, and administered through a transdermal patch.

Nasal administration of the compounds of the invention may also be desired especially for the treatment of common cold and allergic rhinivity.

The present invention also concerns a method for the detection of picornaviral infection. According to the method of the invention a compound of the invention bearing a detectable label (for example attached to R^2), is incubated with a sample, suspected of containing picornaviruses, under conditions enabling binding of the compound to proteases. Preferably the sample should be treated with a lysing agent in order to release the picornavirus proteins from inclusion bodies. Then it is determined whether the labeled compounds of the invention are bound to any proteins in assay. A positive answer (beyond a predetermined control level) is indicative of the presence of a picornavirus in the assayed sample.

Since the compounds of the invention may be tailored to bind specifically to a desired species of picornavirus, by modifying R^1, it is possible according to the detection method of the invention not only to determine the presence of a virus, member of the picornavirus family, in the sample but also to determine the specific species of the picornavirus to which it is bound.

The present invention will now be further illustrated with reference to the following non-limiting drawings and examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: shows amino acid sequences of oligopeptides capable of forming R^1 in accordance with the second embodiment of the invention. Left column - the name of the virus as depicted by the GCG-code in the database. Right column - the appropriate amino acid sequences in one letter code;

Fig. 2: shows amino acid sequences capable of forming R^2 in compounds of the invention. Left column - the name of the virus as depicted by the GCG-code in the database. Right column - the appropriate amino acid sequences in one letter code;

Fig. 3: shows various compounds for forming R^1 in accordance with the third embodiment of the invention, prepared by peptidomemtic;

Fig. 4: shows reverse phase HPLC results of cleavage of a 3C protease consensus substrate, by Rhinovirus protease 3C in the presence (bottom) and absence (top) of the 3C protease inhibitor DIF1;

Fig. 5: shows the rate of cleavage of a 3C protease consensus substrate by the Rhinovirus 3C protease, as determined by HPLC assay in the absence (■) and presence (▲) of 270μM DIF1 (calculated K_i 0.22 mM); and

Fig. 6: shows cleavage of the substrate N-succinyle 3-Ala-Ala-Ala-p-nitronilide in by elastase (♦), elastase + EtoH (■) and elastase + DIF1 (▲).

DETAILED DESCRIPTION OF THE INVENTION

I. Synthesis of DIF1

Synthesis was carried out according to the procedure of Masento *et al.*, *Biochem. J.*, **25b**, 23-28 (1988)

I. Cleavage assay

The cleaving activity of a Rhinovirus (HRV) 3C protease was determined by employing a synthetic N-acetylated 10-mer peptide possessing the consensus sequence for cleavage by Rhinovirus protease 3C (Queens's University, Ontario) as a substrate. Specific cleavage of this peptide [(N-Ac)Arg-Ala-Glu-Leu-Gln-Gly-Pro-Tyr-Asp-Glu] by HRV 3C provided two pentapeptides. In a typical experiment 80 μg of peptide dissolved in 100 mM TRIS (pH 8) and 100 mM NaCl, was incubated at 35°C with 2.5 μg of HRV 3C protease with and without DIF1, to give a total volume of 30ml. In both cases 3C was suspended in 40mM Tris-HCL, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 2.5 μg of DIF1 (dissolved in 1 μl of Ethanol) were used, corresponding to an inhibitor concentration of 270 μM. Inhibitory effects due to the organic solvent were not observed; nevertheless, 10μl of ethanol were added to the control. Aliquots were drawn at short intervals (within ca. 40 min.) and quenched with an equal volume of 1% TFA in Methanol (HPLC grade).

II. HPLC

The digestion mixtures obtained from the cleavage assay, as described above, were separated by reverse phase HPLC (Pharma Biotech Resourec™ R.P.C. 15μm beads 30 x 6.4 mm) using a 20-80% gradient of 0.1% TFA in MeoH (Carlo-Erba)

in 5 minutes (flow rate of 1 ml/min). Simultaneous detection at 280 and 215 nm provided additional resolution (Pharmacia LKB RSD, Uppsala, Sweden), as only one of the pentapeptide cleavage products retains absorption at 280nm (namely the tyrosine containing GPYDE).

The inhibitory potential was quantitated by integration of peak areas under the 280nm absorption curve. The degree of cleavage was calculated as the ratio of peak areas corresponding to peptide alone and peptide plus GPYDE product. Quantification was based on the linear (less than 20% cleavage), initial portion of the activity vs time data.

Example 1: Inhibition of 3C activity by DIF1

Fig. 4 shows the HPLC results of the digestion mixture at several time points of incubation with 270 μm DIF1 and in its absence. Arrows indicate the original 10mer synthetic substrate and the two pentapeptides which are the digestion products. As can be seen, in the absence of DIF1 there was a marked decrease in the level of the original substrate and a marked increase in the level of its digestion products as compared to corresponding results in the presence of DIF1, indicating that DIF1 has an inhibitory effect on the cleaving activity of the 3C protease.

Fig. 5 shows a marked decrease in the percentage of the substrate cleaved, as a function of time in the presence of 270μM DIF1 as compared to control, carried out under the same condition as described above. Ki was calculated to be 220μM.

Example 2: Specificity experiments

A. Cleavage assay - elastase

The cleavage activity of elastase was determined in 0.1M Tris HCl pH 7.0 buffer containing 20μg/ml enzyme and its synthetic substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide.

Results

As can be seen in Fig. 6 50mM of DIF1 did not inhibit the cleavage activity of elastase, indicating the DIF1 is not an inhibitor of elastase.

B. Cleavage assay - Cathepsin B, Chymotripsin, Papain and Ficin

All enzymes and substrates were purchased from Sigma.

Cathepsin B was assayed according to the method of Bajowski and Frankfater (*Anal. Biochem.* **68**:119 (1975)).

Chymotripsin was assayed in 0.1M KPi buffer pH 7.0 containing 0.04 μg/ml enzyme and 200μM N-benzoyl-L-tyrosine ethyl ester (from a 20mM stock in dimethyl sulfoxide). The reaction was monitored by spectrophotometry at 256 nm.

Papain and Ficin were incubated for one hour in an activation solution containing 0.1 M KPi buffer pH 7.0, 0.5 mM cystein, 20mM EDTA and 65 μg enzyme. The assays were carried out by adding 20μl aliquots from the activation mix to 0.1 M KPi buffer pH 7.0, containing 12.5 nM N-CBZ-Gly-p-Nitrophenyl ester. The reaction was monitored by spectrophotometry at 405 nm.

Results

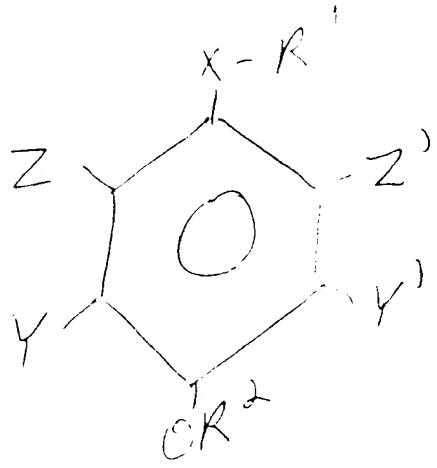
All DIF1 concentrations tested were found not to effect the protealytic activity of Cathepsin B, Chymotripsin, Papain and Ficin (data not shown).

Example 3: Inhibition by derivatives of DIF1

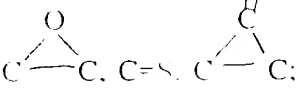
The inhibitory activity of two derivatives of DIF1 of formulae III at IV was determined as described above for DIF1. Ki for the compounds of formula III and IV was found to be 150μM and 154μM respectively, showing that derivatives of DIF1 may feature a better inhibitory activity than the native compound.

CLAIMS

1. A specific inhibitor of picornavirus 3C and / or 2A proteases having the formula:



I

Wherein X is C=O, S=O,  , C=S, ;

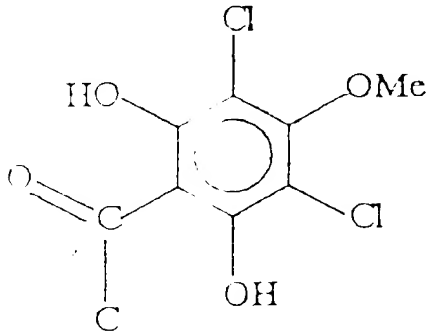
R^1 is a hydrocarbon group, an oligopeptide of 3 to 12 amino acids, or a small organic molecule prepared by peptidomimetic, having the same binding properties as said oligopeptide;

R^2 is H, C_1-C_{12} substituted or unsubstituted hydrocarbon, or an oligopeptide of 3 to 12 amino acids;

Z and Z^1 are each independently OH, SH or one of Z and Z^1 may also be H;

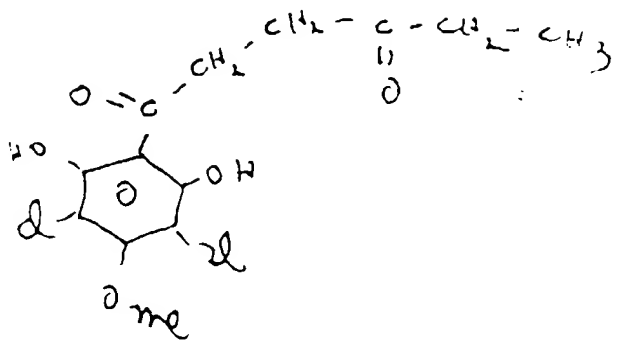
Y and Y^1 are the same or different electron withdrawing groups or one of Y and Y^1 may also be H.

2. An inhibitor according to claim 1 being differentiation inducing factor 1 (DIF1) of the formula:



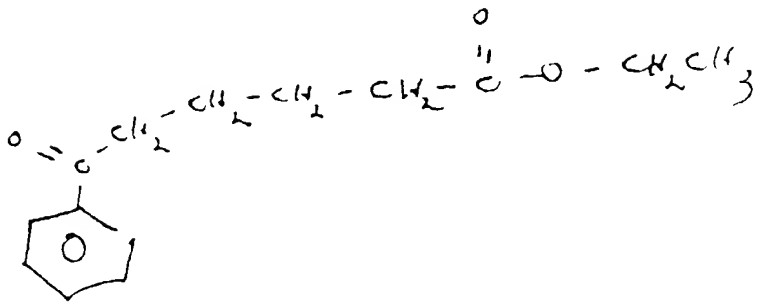
II

3. An inhibitor according to claim 1 of the formula:



III

4. An inhibitor according to claim 1 of the formula:



IV

5. A pharmaceutical composition comprising as an active ingredient a therapeutically effective amount of the inhibitor of claims 1 to 4 and a pharmaceutically acceptable carrier.

6. A pharmaceutical composition according to claim 5 for the treatment of picornaviral infections.

7. A pharmaceutical composition according to claim 6 for the treatment of a disease selected from the group consisting of:

common colds, allergic rhinitis, poliomyelitis, hepatitis-A, encephalitis, meningitis, foot-and-mouth disease and encephamiocarditis

8. A method for the treatment of picornavirus infections by administering to a subject in need of such treatment of a therapeutically effective amount of the inhibitor of claims 1 to 4.

9. A method for determining the presence of picornavirus is a sample comprising:

(a) conjugating the inhibitor of claims 1 to 4 to a detectable label;

(b) contacting the labeled inhibitor with the sample under conditions enabling binding between the inhibitor and viral proteins;

(c) determining whether any proteins in the sample are bound to the inhibitor, a positive answer indicating the presence of picornaviruses in the sample.

For the Applicants
REINHOLD COHN AND PARTNERS
By :

R1

The 12 amino acids which follow the 2c/3d cleavage site

POLG_TMEVB	SPPDWEHFENIL
POLG_TMEVD	SPPDWEHFENIL
POLG_TMEVG	SPPDWQHFENIL
POLG_EMCVB	APVDEVSFHSVV
POLG_EMCVD	APVAEVSFHSVV
POLG_EMCV	GPVDEVSFHSVV
POLG_HRV1B	GPISLDAPPPPA
POLG_HRV2	GPIDMKNPPPPA
POLG_HRV89	GIDLQSPPPPAI
POLG_POL1M	GPLQYKDLKIDI
POLG_POL1S	GPLQYKDLKIDI
POLH_POL1M	GPLQYKDLKIDI
POLG_POL3L	GPLQYKDLKIDI
POLG_POL32	GPLQYKDLKIDI
POLG_COXA2	GPLRYKDLKIDV
POLG_COXA4	GPIQYRDVMIDI
POLG_SVDVH	GPPVYREIKISV
POLG_SVDVU	GPPVYREIKISV
POLG_COXB1	GPPIYREIKISI
POLG_ECL1G	GPPIYREIKISV
POLG_COXB5	GPPIYREIKISV
POLG_COXB3	GPPVYREIKISV
POLG_COXA9	GPPIYREIKISV
POLG_COXB4	GPPVYREIKISV
POLG_HUEV7	GPPTFKEIKISV
POLG_HRV14	GPVYKDLEIDVC
POLG_BOVEV	GPVCYKPLRIEV

Fig 1

Fig 2

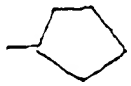
R^2

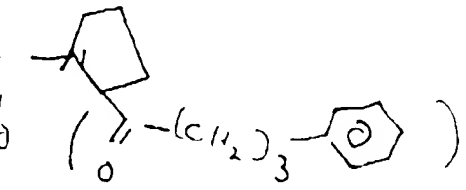
The 12 amino acids which precede the 2c/3a cleavage site

POLG_TMEVB	ENVKKMNSLVAQ
POLG_TMEVD	ENVKKMNSLVAQ
POLG_TMEVG	ENVKKMNSLVAQ
POLG_EMCVB	KVLTTVQTLVAQ
POLG_EMCVD	KVLTTVQTLVAQ
POLG_EMCV	KVLTTVQTLVAQ
POLG_HRV1B	RQVVDVMSAIFQ
POLG_HRV2	RQVVDVMTAIFQ
POLG_HRV89	SSAAQAMEAIFQ
POLG_POL1M	SNIGNCMEALFQ
POLG_POL1S	SNIGNCMEALFQ
POLG_POL1M	SNIGNCMEALFQ
POLG_POL3L	SNIGNCMEALFQ
POLG_POL32	SNIGNCMEALFQ
POLG_COXA2	ANIGNCMEALFQ
POLG_COXA4	ANIGNCMEALFQ
POLG_SVDVH	HSVGATLEALFQ
POLG_SVDVU	HSVGATLEALFQ
POLG_COXB1	HSVGATLEALFQ
POLG_EC11G	HSVGATLEALFQ
POLG_COXB5	HSVGATLEALFQ
POLG_COXB3	HSVGTTLEALFQ
POLG_COXA9	HSVGATLEALFQ
POLG_COXB4	HSVGATLEALFQ
POLG_HUEV7	NSTQDKLEALFQ
POLG_HRV14	MQITDSLETLFQ
POLG_BOVEV	YNIGNVLEALFQ

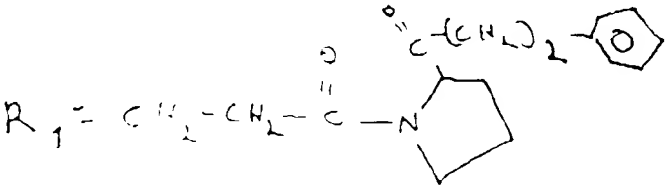
Fig 3

R_1 : Peptidomimetics (examples)

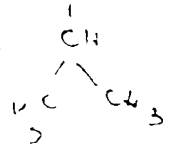
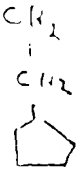
1. $R_1 = CH_2-C_6H_4-(CH_2)_n-C(=O)-R'$ $n = 1, 2$ $R' = CH_3$, OEt,  , NH_2

2. $R_1 = CH_2-CH_2-C(=O)-$  or $(CH_2)_4-C_6H_5$

$R_1 = CH_2-CH_2-C(=O)-CH_2-CH_3$ (the first derivative that we reached)

$R_1 = CH_2-CH_2-C(=O)-N$ 

$R_1 = -NH-CH(R'')-C(=O)-CH_3$

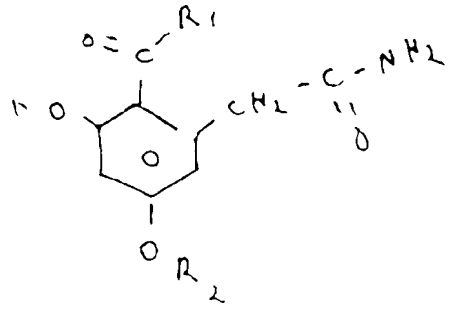
R'' =  , 

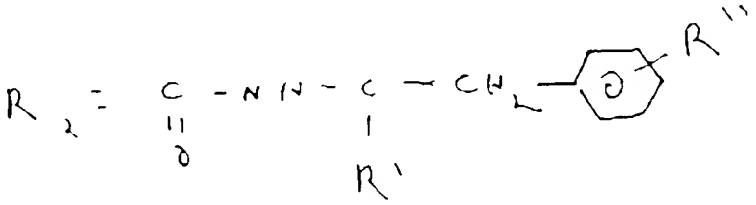
$R_1 = CH_2-C(R')(=O)-NH-CH(R''')-C(=O)-CH_3$

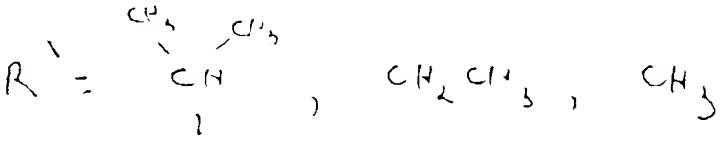
R''' = aromatic ring or

Fig 3

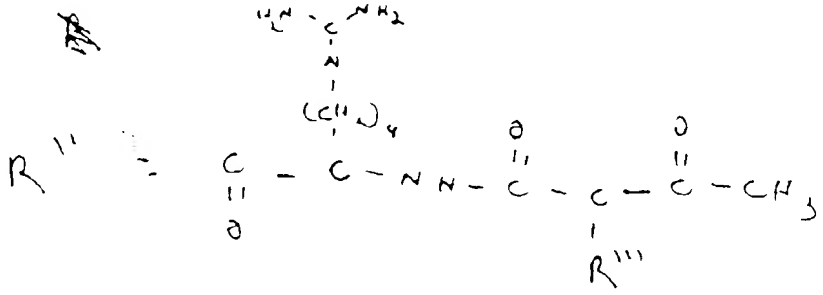
3. modification on OH site according to modeling:







or any short branched alyphatic chain

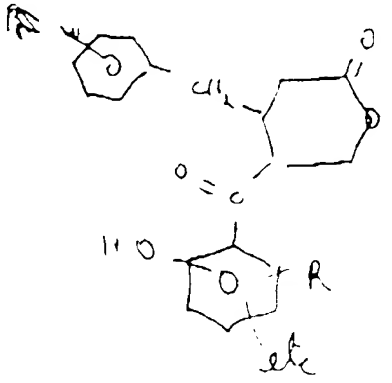


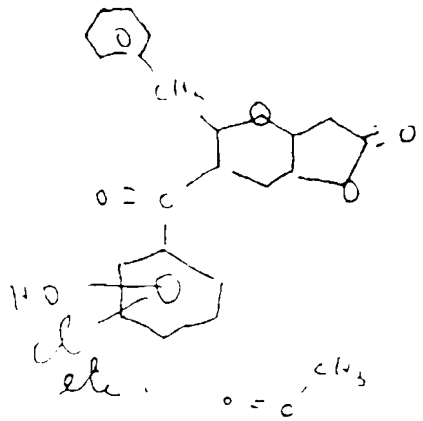
R''' = small or branched aliphatic like side chains of: leu, val, ile, ala

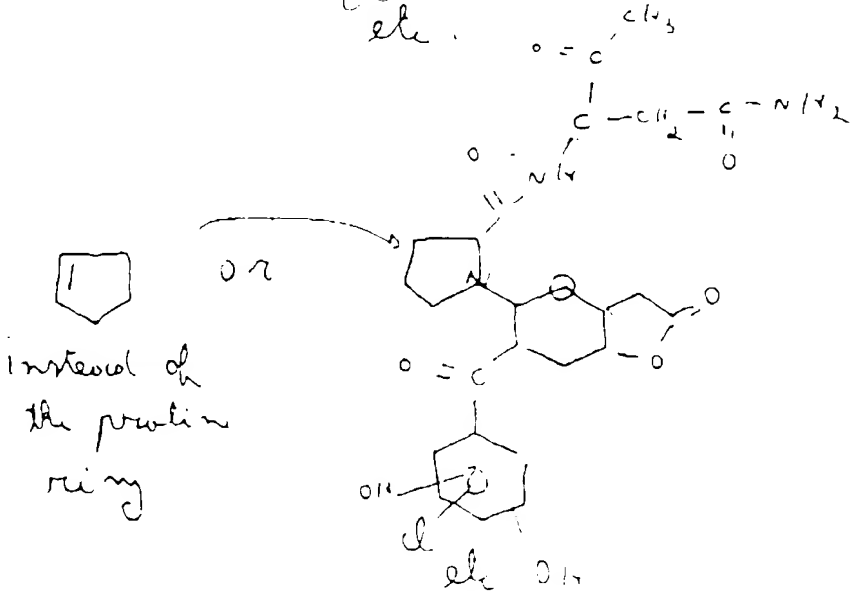
(continue)

Fig 3

other R1 (peptida mimetics.







(continue)

Fig 4

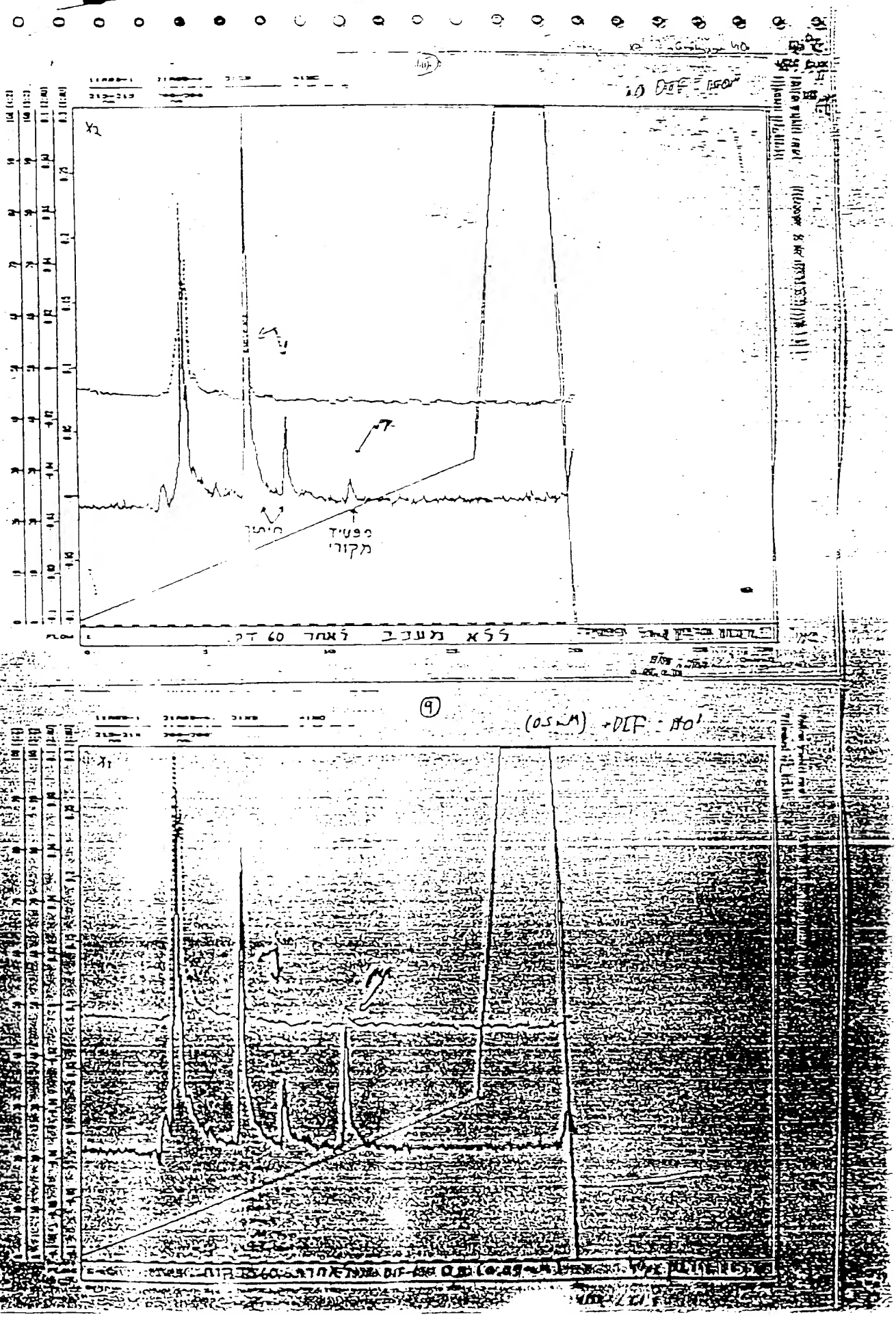


Fig 5

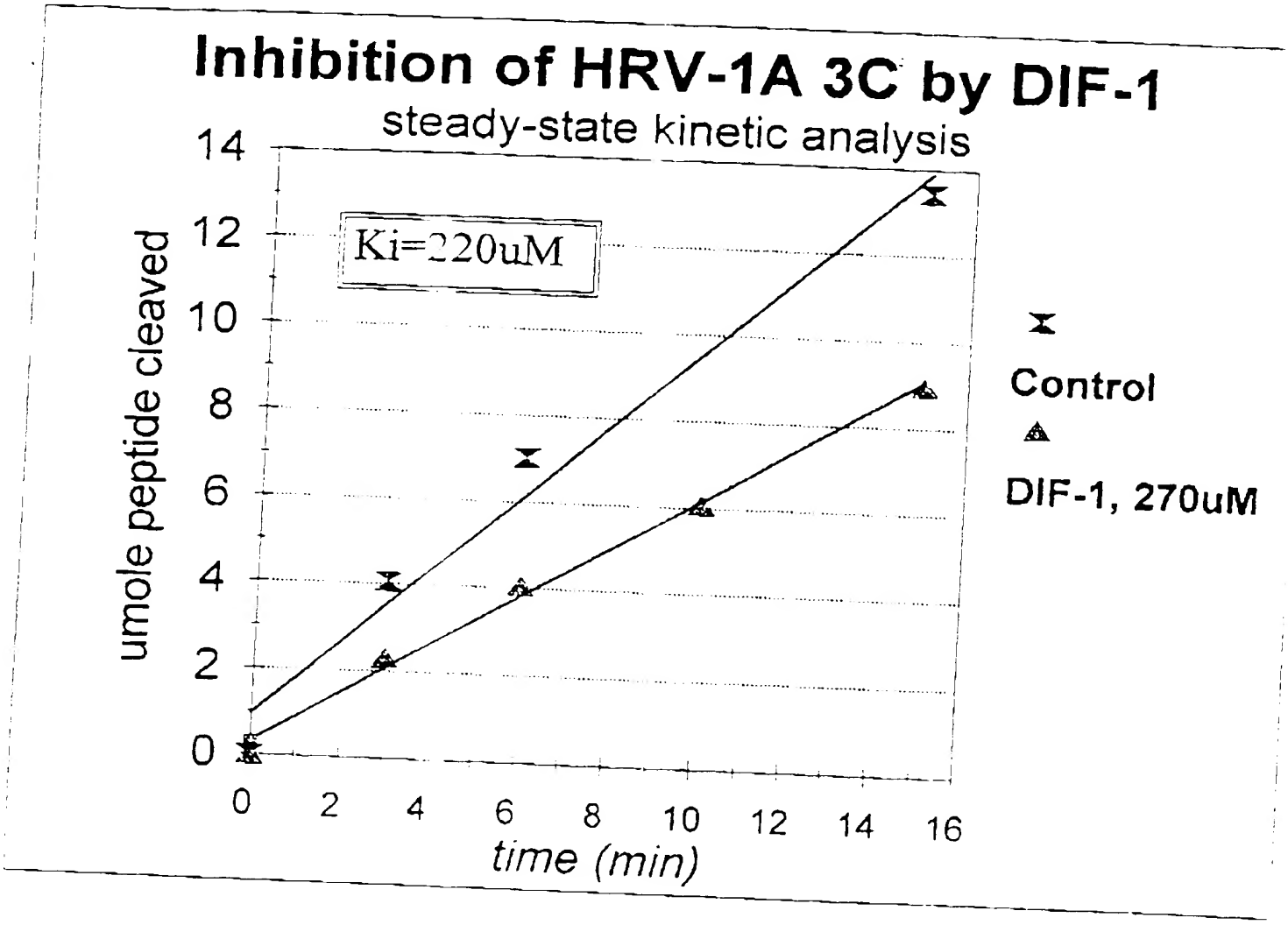


Fig 5

Ki_app = [I]/(Vc/Vi-1) ; Ki = Ki_app/(1+[S]/Km)

attempted inhibition of elastase by DIF-1, 50 mM cond

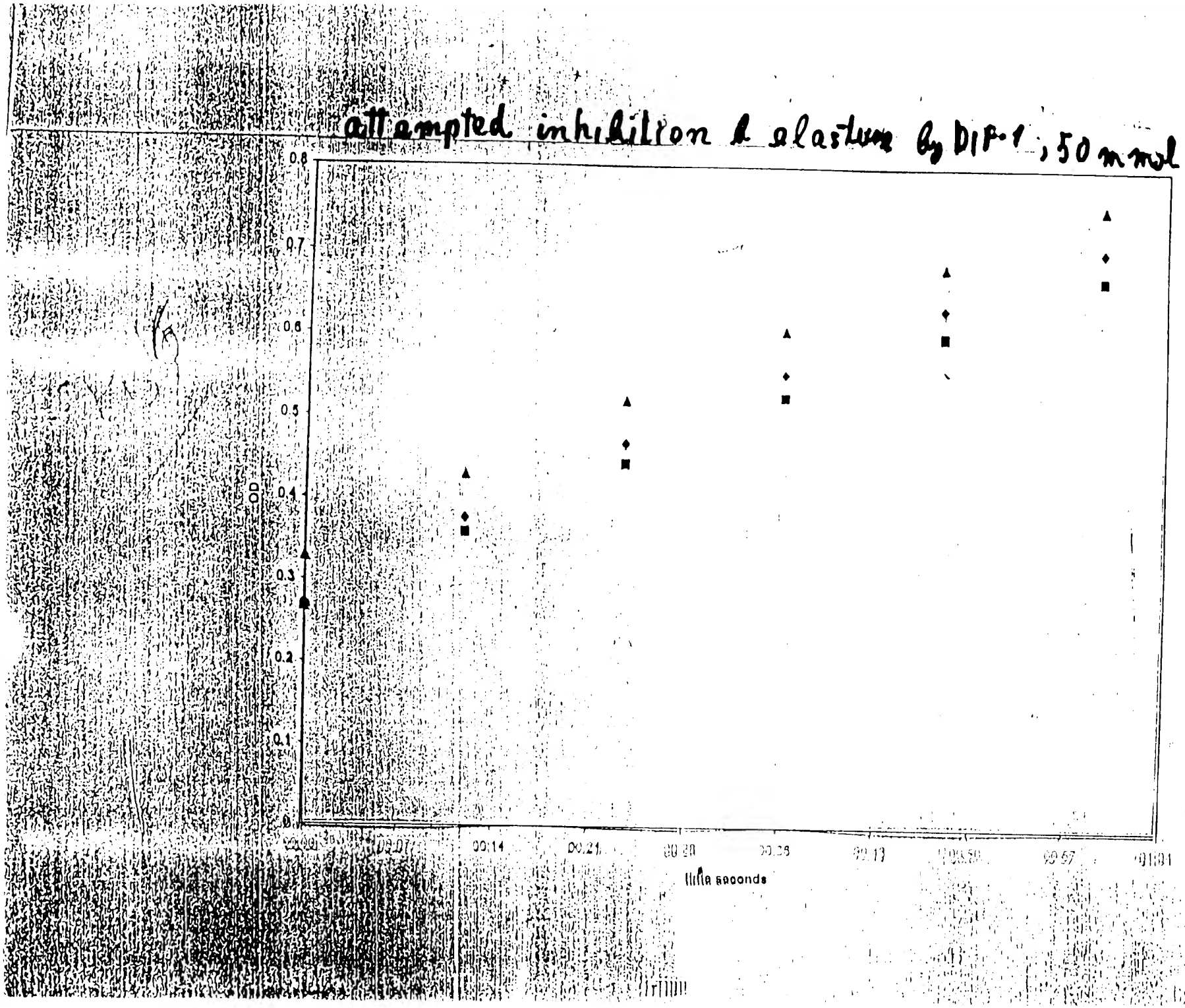


Fig 6